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This project demonstrates the use of capillary electrophoresis as an improvement to existing methods of analysis for carbon monoxide (CO) in human hemoglobin (Hb). Current methods for CO analysis are CO-oximetry and tonometry/gas chromatography, which involve lengthy sample manipulation followed by spectroscopic or gas displacement measurements. These methods are time-consuming and technically difficult because of the many manipulations they require. Capillary electrophoresis (CE), a modern analytical technique, is presented as a faster and easier method to quantify CO in Hb. Several steps towards the develoipment of a CE procedure to detect CO in blood have been completed. The first step of this analysis involved isolating heme, the CO binding site in Hb, from the Hb molecule. Initially, mixtures of reduced heme and heme-CO were successfully isolated from Hb standards and separated using CE. Finally, reduced heme and heme-CO were isolated from three blood samples of CO-related accident victims and analyzed using CE. Differences in the heme-CO signals from blood samples known to contain fatal and non-fatal levels of CO were observed. These differences were quantified by measuring the areas under the peaks representative of reduced heme and heme-CO. Results indicate that an improvement was made to the current methods of CO analysis.

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DEVELOPING METHODS IN CAPILLARY ELECTROPHORESIS FOR THE DETECTION OF CARBON MONOXIDE POISONING

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ABSTRACT

This project demonstrates the use of capillary electrophoresis as an improvement to existing methods of analysis for carbon monoxide (CO) in human hemoglobin (Hb).

Current methods for CO analysis are CO-oximetry and tonometry/gas chromatography, which involve lengthy sample manipulation followed by spectroscopic or gas displacement measurements. These methods are time-consuming and technically difficult because of the many manipulations they require. Capillary electrophoresis (CE), a modern analytical technique, is presented as a faster and easier method to quantify CO in Hb.

Several steps towards the development of a CE procedure to detect CO in blood have been completed. The first step of this analysis involved isolating heme, the CO binding site in Hb, from the Hb molecule. Initially, mixtures of reduced heme and heme-CO were successfully isolated from Hb standards and separated using CE. Finally, reduced heme and heme-CO were isolated from three blood samples of CO-related accident victims and analyzed using CE. Differences in the heme-CO signals from blood samples known to contain fatal and non-fatal levels of CO were observed. These differences were

quantified by measuring the areas under the peaks representative of reduced heme and heme-CO. Results indicate that an improvement was made to the current methods of CO analysis.

KEYWORDS Capillary electrophoresis, hemoglobin, carbon monoxide, heme.

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BACKGROUND

Introduction

On April 19, 1989 during a gunnery exercise aboard the USS IOWA (BB-61), the number 2 turret exploded killing 47 crewmen. A postmortem analysis of the bodies was conducted(1). One aspect of this analysis included a spectrophotometric determination of carbon monoxide (CO) levels in the victims' blood using the IL 482 CO-oximeter. Carboxyhemoglobin (Hb-CO) levels found in the victims provided a picture of where they were at the time of the explosion and how they may have died. For example, those victims who were working at or near the turret at the time of the explosion died of blunt force injuries from the explosion. Their corresponding Hb-CO levels were in normal range for humans (0-10%). Other victims' blood samples measured Hb-CO levels greater than 28%. Their bodies were recovered from areas removed from the immediate site of the explosion. This Hb-CO determination indicates that they were alive to take several breaths after the explosion occurred. The state of these recovered bodies also indicated that they died from a combination of blunt force trauma and thermal injuries. Those individuals whose bodies were not burned or mangled measured

Hb-CO levels from 60 to 90%. It was determined that they were most removed from the explosion and had died solely from smoke inhalation.

The toxicological findings from the USS IOWA disaster provided helpful insight into the victims' causes of death through body location at the time of the explosion. Such information is sought after any disaster. The results of this type of investigation play an integral role in safety investigations and future legal proceedings. The following report reveals current developments made towards an improved method for gathering this type of toxicological information to answer the questions that such accidents present.

History

Concentrating on methods and modifications for the analysis of carbon dioxide (CO_2), oxygen(O_2), and CO content, Van Slyke pioneered manometric methods for measuring gases in blood (2). Historically, the Van Slyke and gas chromatographic (GC) methods have given the greatest precision and accuracy with regard to CO analysis. More recently, high performance liquid chromatography (HPLC), infrared gas analysis, and other spectrophotometric techniques have been employed (2).

The method currently used for determination of Hb-CO

levels involves a combination of spectroscopic and gas chromatographic methods. Hb-CO levels are first determined by a spectrophotometric analysis with the IL 482 CO-oximeter (or a similar instrument)(3). The CO-oximeter measures the concentration of CO in a sample based on the Hb-CO molecules' absorption of visible light of a specific wavelength. The level of absorption is directly proportional to the concentration of Hb-CO in the sample. Therefore, by monitoring a sample at a particular wavelength of absorption, the concentration of CO present in that sample can be measured. Confirmation of these spectrophotometric results follows with tonometric/GC analysis (4). This analysis compares the absorption of visible light by CO from an untreated blood sample to that of a blood sample that has been completely saturated with CO gas. The saturated sample acts as the reference to which the untreated blood sample is compared. From these results, a relative % Hb-CO level in the blood sample is determined.

Although these methods have proven helpful in determining Hb-CO levels in blood, they are not reproducible. Often, a tonometric/GC confirmation results in a different Hb-CO level than that determined by the CO-oximeter. Historically,

coefficients of variation have reached only as low as 6% for these techniques. The magnitude of this error can interfere with the prognosis and treatment in patients or in determination of cause of death (5). For example, Hb-CO levels of greater than 15% characterize a severe case of CO poisoning, while an average smoker may already have up to 10% Hb-CO in his blood. As this example indicated, a new, more accurate technique for CO analysis is necessary. I propose capillary electrophoresis (CE).

Capillary Electrophoresis

CE is an extremely efficient separation technique in which solutes, under the influence of an electric field, travel through a narrow-bore, fused-silica capillary tube (typically having an inner diameter of 50 μm). Separation of the solutes occurs as a result of the solutes' different rates of migration through the capillary. The magnitude of these mobilities depends on the size, charge, and conformation of each solute(6). For instance, a molecule with a +2 charge will travel faster towards a negatively charged electrode than one with a +1 charge. Heavier molecules will travel more slowly through the column. Also, a more planar molecule will travel at a slower rate through the capillary due to its

larger surface area.

There are four essential components in any CE system (see Figure 1). A high voltage source is connected to two vials by means of platinum electrodes. The vials, and a capillary that is suspended between the vials, are filled with buffer, which is a solution composed of positively and negatively charged particles (ions). Upon application of a high voltage, the buffer migrates from one end of the capillary to the other as a result of the field within the capillary (6,7). This migration is termed electroosmotic flow (EOF). The EOF, combined with the inherent electrophoretic mobilities of the species of interest, will carry the solutes through the capillary and past an optical window where their presence can be detected in various ways. The most widely used methods of detection include monitoring solutes' absorbance of light in the ultraviolet or visible range or their emission of light (fluorescence/phosphorescence) following absorption. The signal that each solute produces is called a peak. A readout containing peaks is generated from which the amount of time different solutes spend in the capillary (retention time) can be determined. A difference in peak retention time of

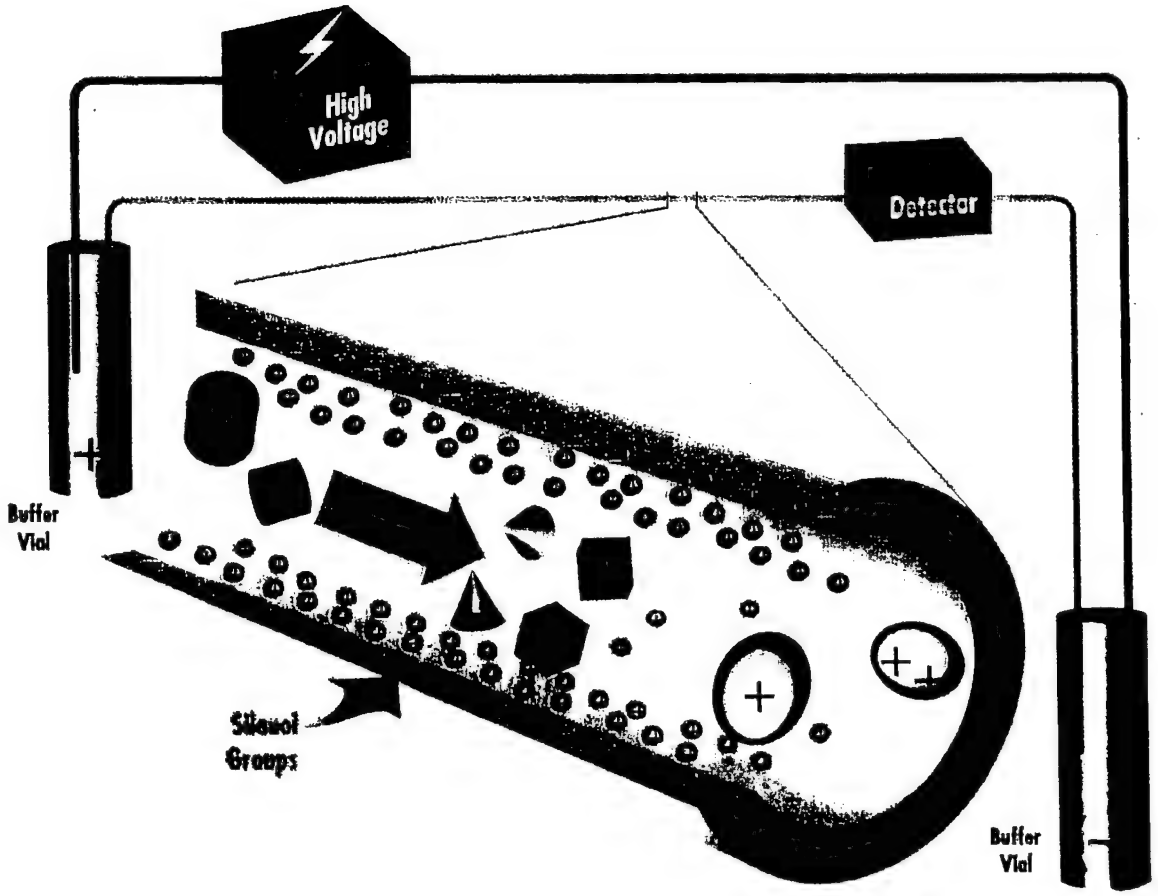

Figure 1: A block diagram of a basic capillary electrophoresis system. The magnified view depicts the capillary interior with molecules of different size and charge being separated. (Courtesy of Beckman Corporation)

~0.5 minutes indicates successful separation of solutes in CE. The height of the peak is measured in arbitrary absorbance units. The area under each peak is a direct indication of the amount of solute present.

CE is a revolutionary, and relatively new, technique that is being developed to quantitatively and qualitatively analyze biological samples. Unlike many current methods of analysis, CE often does not require lengthy sample preparation prior to analysis, which reduces sample loss and analysis time. Because of its relatively low operating cost and its reproducibility, CE offers a highly efficient method of analysis. Its ability to resolve components in a mixture surpasses most modern analytical separation techniques. Also, CE separations require minute amounts of sample. This is advantageous, for in many cases there is very little sample to analyze. In the case of a fire victim suspected of dying from overexposure to CO gas, there are often only small amounts of blood present.

One phenomenon that must be considered in CE analyses is that many macromolecules, such as Hb, tend to stick to the capillary wall. This adsorption results in decreased resolution (peak separation) through diminished efficiency

(sharpness of the peak). One can attempt to control this adsorption using several techniques. Column coating and pH adjustment are the most common methods employed to eliminate this problem.

Coating a capillary with polymers, such as polybrene or polyacrylamide, before injection of the solute will deactivate the surface of the wall, thus minimizing adsorption (8, 9). By lowering the pH of the CE separation buffer, the charge of the capillary wall can be made more positive, so that it repels a positively charged solute. Although the use of pH extremes is very effective in reducing ionic interactions between the capillary walls and the solute, the magnitude of the EOF is greatly reduced at lower pH, thus increasing analysis time (6,7). However, pH manipulations may favorably affect the mobility of the molecules by causing changes in their steric configurations (10). Sometimes, a difference between two molecules' steric configurations is the only characteristic that will cause them to separate in a CE column. An example of such a difference is that between reduced heme and heme-CO.

Hemoglobin: Structure and Function

Hb is the protein found in blood that is responsible for

the transport of O_2 and CO_2 throughout the body. Hb, with a molecular weight (MW) of 64,450 Daltons, is an extremely large molecule. It is a tetramer composed of two α and two β protein chains. Within each of these chains rests a heme group (MW 616 Daltons). The carbon and nitrogen atoms of the heme group are depicted in Figure 2 as green and purple spheres. The central iron atom, which is the actual site for O_2/CO_2 binding, is represented by a pink sphere. Its essential biological function makes Hb a very important molecule to study. Hb can also bind to other dissolved gases, such as CO and cyanide (CN), which are harmful, or even fatal, depending on the amounts present in the blood. Circumstances inducing CO poisoning can leave little time for analysis. Therefore, a quick, reliable analysis of Hb or heme for the presence of CO is advantageous. Also, accurate determinations of cause of death due to CO poisoning are sometimes necessary (11, 12).

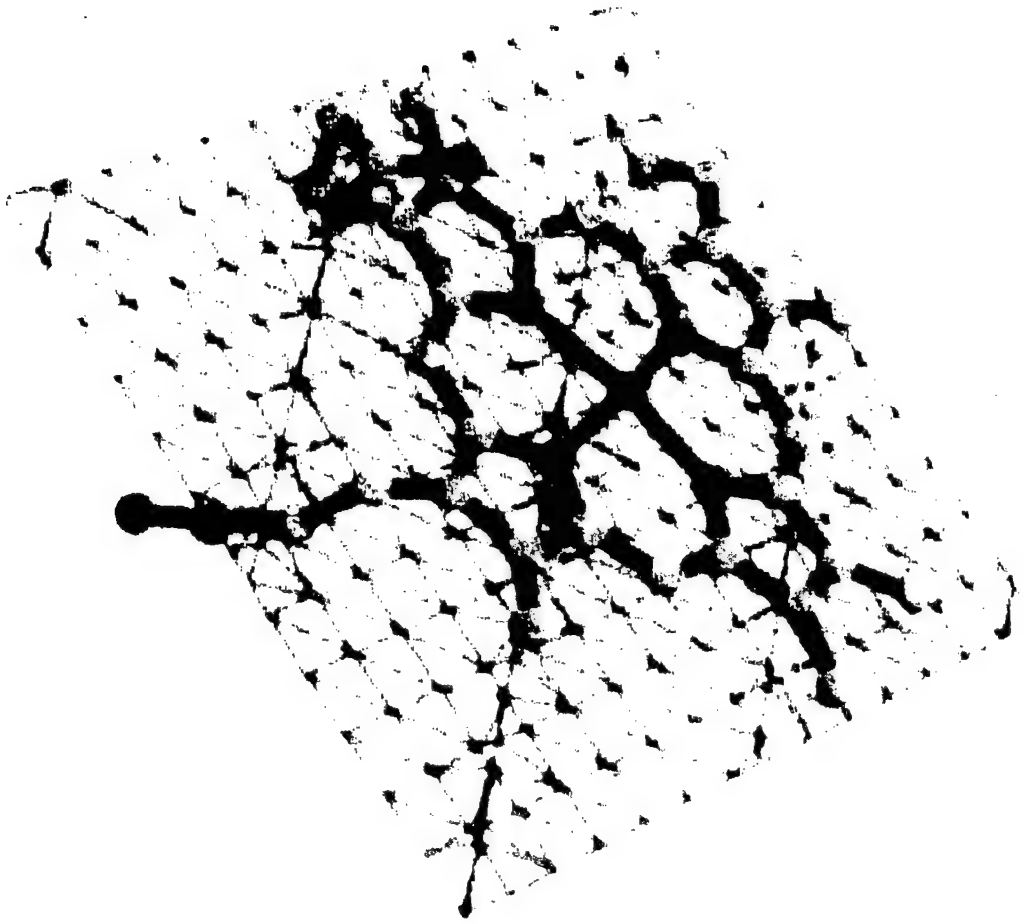

Figure 2: A best-fit plane depicting all the atoms of the heme group. The equation of the plane was computed by program PRINCIP and its surface generated by SURFNET; it is rendered here by Raster3D.

EXPERIMENTAL SECTION

Materials

Ultra high purity solvents were obtained from the United States Naval Academy chemistry department. Human Hb and hemin standards were purchased from Sigma Chemical Company (St. Louis, MO). Sodium bisulfite, blood samples, and IL 482 CO-oximeter diluent were obtained from the Armed Forces Institute of Pathology (AFIP) (Rockville, MD). The IL 482 CO-oximeter diluent consisted of 100 g/L octylphenoxypolyethoxyethanol. The buffer used in this experiment was a solution of 0.01 M Na_2HPO_4, 0.006 M $Na_2B_4O_7$, at various pHs. HCl (36% w/w) and 0.01 M NaOH were used to alter the pH of the buffer. The running buffer used for successful isolation and separation of reduced heme and heme-CO had a pH of 9.2. Capillary columns were purchased from Polymicro Technologies (Phoenix, AZ).

Apparatus

An HP 5890 Gas Chromatograph with a Tekmar 7000 headspace sampler was used for the tonometry/GC analyses. An IL 482 CO-oximeter was used to analyze Hb-CO using CO-oximetry. The CE apparatus principally used in this experiment consisted of a Spellman CZE1000R high voltage power supply, a narrow-bore, fused-silica capillary column, and microcentrifuge buffer

vials with platinum electrodes connected to the high voltage source. Typically, the capillary columns were 80 cm in length (50 cm to detector) and had a 50 μm inner diameter. Detection was performed at 390 nm using an ISCO Model 3140 multivariable wavelength detector. Initial studies of Hb were performed on a Waters Quanta 4000 CE, a self-contained instrument, with polybrene-coated and polyacrylamide-coated columns and detection at 214 nm.

Methods of Analysis

CO-oximetry/tonometry/GC Comparison Study

Before embarking on developing a new method of analysis for CO poisoning, it was important to have a working knowledge and understanding of the drawbacks of the current methods. The degree of precision of the CO-oximetry and tonometry/GC methods of analysis was determined using thirteen blood samples of victims in CO-related incidents. They were prepared in accordance with the protocol for the IL 482 CO-oximetry and tonometry/GC analyses provided by AFIP (3,4).

For the CO-oximetry analysis, 500 μL of CO-oximeter diluent was pipetted into a tube with 500 μL of a blood sample. The diluent hemolyzed the blood, causing the Hb to be released from the blood cells. Sodium bisulfite (50 mg/mL)

was added to the sample to reduce the Fe in each Hb molecule to the Fe^{2+} oxidation state and to strip each heme group of all ligands except CO. This solution was centrifuged at 10000 rpm and the isolated layer containing the heme was inserted into the CO-oximeter for a spectrophotometric quantification of Hb-CO.

The tonometry/GC analysis was used to confirm the results of the CO-oximeter analysis. In the tonometry/GC analysis, 2.0 mL of blood was added to 1.0 mL of diluent solution in a test tube. A 1.0 mL aliquot of this solution was sealed in another test tube and acted as the "untreated" sample for the analysis. A 1.5 mL aliquot was saturated with CO by bubbling the gas into the sample using a tonometer. The sample in this tube was sealed and acted as the "saturated" sample. In the tonometric/GC analysis, $K_3Fe(CN)_6$ was added to each sample. The CN, which has a similarly strong affinity for heme, dissociates from $K_3Fe(CN)_6$ in solution and replaces the CO molecules attached to each Hb molecule, therefore releasing the CO into the headspace of the sealed test tube. A known amount of gas from the headspace was injected into a GC and the amount of CO in this gas sample was determined using the saturated sample as a reference. This amount is directly

related to the amount of CO in the blood sample.

Hemoglobin Studies Using Capillary Electrophoresis

Sample preparation is the first step in any chemical analysis. It was noted earlier that the initial step in this analysis was the isolation of Hb from blood. However, before studies of actual biological samples were performed, pure standards had to be analyzed. These offered the smallest number of variables to interfere in the matrix. For this experiment, an HIV/Hepatitis-tested human Hb standard was used.

An Hb standard (1.0 mg/mL) was prepared by dissolving 10 mg Hb in 10 mL deionized H_2O in a volumetric flask. The standard was hydrostatically injected into an uncoated silica capillary tube (50 μm inner diameter, 50 cm to detector). This injection method operates under the same principles as a siphon, where gravity pulls the standard into the capillary column in the form of a plug. The length of plug introduced into the column is directly proportional to the length of time and the height of the inlet end (relative to the outlet end) of the capillary. Typically, injections were made 15 cm above the outlet buffer vial for 30 seconds and electrophoresed at 20 kV in a 0.01 M Na_2HPO_4 buffer (pH 2.5).

Several attempts to secure a consistent signal for Hb were made. These attempts involved varying the nature of the column walls to prevent these macromolecules from sticking. The Hb standard was run through a column that was dynamically coated with a polymer (polybrene). This procedure involved dissolving polybrene in the buffer so that the column would be coated during the run (8). Attempts were also made to coat the column with a permanent polyacrylamide coating (9). This static coating procedure is applied prior to a run rather than during one.

Heme Studies Using Capillary Electrophoresis

As the goal of this analysis was to study levels of CO poisoning, it was logical to isolate the heme groups, the CO binding site in Hb, from the Hb molecule, and then to analyze them. Each heme group is much smaller than a Hb molecule (heme having a MW of 616 Daltons and Hb 64,450 Daltons). Therefore, the difference in molecular weight between heme and heme-CO is much more significant than between Hb and Hb-CO. Because migration depends on size and charge, a difference in retention times between these two heme species would be more likely to be seen in a CE analysis.

There are several methods of isolating heme from Hb (13).

An acidic acetone extraction was selected because of its ease and availability. A 10 mL aliquot of a 10 mg Hb/mL H_2O standard was prepared. A 100 mL sample of acidic acetone (2% HCl (36% by volume)/98% acetone) was added to this 10 mL Hb standard. The acidic acetone separated the globin chains from each other and the heme groups and caused the chains to precipitate out of solution. This process left the heme groups virtually isolated in the acidic acetone layer. To recover the heme, the solution was centrifuged and filtered to remove the precipitated globin chains from the heme-containing liquid layer.

With a boiling point of 56°C, acetone is a relatively volatile solvent. When exposed to the high voltages required for CE, it will vaporize in the column and result in a failed run. Therefore, to analyze the heme standard using CE, most of the acetone was evaporated from the solution. However, because heme is insoluble in water and soluble in acetone, some of the solvent was left in the solution. Therefore, it was important to roto-evaporate acetone from the sample until heme was observed precipitating out of the solution. Once reduced in volume, the heme sample was diluted in buffer 1:4, thus redissolving the heme prior to analysis.

Another experimental concern was that heme can exist in a reduced or an oxidized state. In the reduced state, the heme's central Fe atom has a charge of +2 and will bind to gas molecules. The reduced heme most likely exists as heme-O_2 which strongly resembles heme-CO in molecular mass and configuration. For this experiment, since a way to differentiate between reduced heme and heme-CO was sought, it was imperative that the heme exist in the reduced state unbound to a gas molecule. This reduced state can be achieved by introducing a strong reducing agent, sodium bisulfite, to the matrix. Sodium bisulfite (50 mg) was added to reduce each mL of heme sample. The sample was then centrifuged and the liquid layer was collected by filtering. This reduced, deoxyheme-containing liquid layer was diluted in degassed buffer at a 1:4 ratio prior to CE analysis and used as the standard for analysis.

Blood Studies Using Capillary Electrophoresis

Human blood samples were repeatedly analyzed over a several-day period for heme-CO and reduced heme levels. Prior to the extraction of heme from a blood sample, it was necessary to break open, or lyce, the blood cell walls with a solvent in order to release the Hb molecules. This was done

by adding 5 mL of CO-oximeter diluent to 5 mL of blood sample. This 1:1 solution was centrifuged and the isolated liquid layer was collected for the acetone extraction. The procedure for the acetone extraction was identical to that described above.

The heme isolated from the blood samples was electrophoresed under the same conditions as those reported for the Hb standards.

Safety Considerations

Action was taken to ensure safety in the laboratory during this experiment. The blood samples and Hb standards had all tested negative for HIV/AIDS and for Hepatitis. Nonetheless, protective gloves, goggles, and a laboratory apron were worn while handling all samples. The lab was scrubbed with a 50% Chlorox solution following experiments that involved these blood samples and biological waste was disposed of properly.

RESULTS AND DISCUSSION

CO-oximetry/tonometry/GC Comparison Study

The results obtained from CO-oximetry and tonometry/GC analysis of 13 blood samples were compared (see Table I). The results from CO-oximetry and tonometry/GC experiments differed by more than 20% for several of the blood samples tested. These differences seem to be real as the protocols for these methods were carefully followed. This comparison study indicates that a new method of analysis for CO poisoning is necessary. This project proposes CE.

Hemoglobin Studies Using Capillary Electrophoresis

Broad signals from Hb were detected at 214 nm by electrophoresing samples of Hb standard at 20 kV in a 0.01 M Na_2HPO_4 buffer (pH 2.5). However the retention times of these signals were not reproducible. Many times the Hb adhered to the column, resulting in a failed run. Further attempts were made by running the Hb standard with a buffer of a higher pH (i.e. 7.4, the physiological pH) and at varied voltages. These variations only resulted in further inconsistent or failed runs.

TABLE I: Comparison Study of CO-oximetry and Tonometry/Gas Chromatography of 13 Blood Samples of Victims of CO-related Incidents

Sample #	% Hb-CO from CO-oximetry	% Hb-CO from Tonometry/GC	Difference in % Hb-CO[1]
1	84.1	80.24	**3.9**
2	0.100	0.6800	**0.6**
3	78.3	50.95	**27.4**
4	79.4	80.36	**0.96**
5	51.1	31.70	**19.4**
6	34.7	22.15	**12.6**
7	89.8	67.18	**22.6**
8	81.5	76.30	**5.2**
9	73.5	82.00	**8.5**
10	73.7	54.98	**18.7**
11	68.0	44.69	**23.3**
12	37.6	30.41	**7.2**
13	74.6	76.35	**1.85**

[1]These values are the absolute difference between the % Hb-CO values provided by the CO-oximetry and tonometry/GC analyses.

With a static polyacrylamide coating to reduce adsorption to the column walls, reproducible signals for Hb (20 kV, 0.01 M Na_2HPO_4, pH 2.5) were observed. After obtaining a consistent signal for Hb, the Hb sample was saturated with CO prior to injection. This saturation step required bubbling CO gas through the sample for 2 minutes (4). Under the same conditions as those used to analyze Hb, a reproducible signal for Hb-CO was obtained. However, there was no difference in the retention times measured for Hb and Hb-CO. These results led to the following conclusion. Hb is a very large molecule with four receptor sites (heme groups) to which CO can bind. When CO (MW 28 Daltons) binds to Hb, it increases the molecular weight of the molecule by 112 Daltons. This change is not significant enough to provide a measurable difference in the migration rates of Hb-CO and Hb. Furthermore, when gas molecules bind to Hb, the conformation of the molecule changes. However, under the above conditions, this change was also not large enough to use CE to distinguish between the Hb and Hb-CO (i.e. the retention times of the two species were indistinguishable).

Heme Studies Using Capillary Electrophoresis

Heme was reduced with sodium bisulfite to remove all

attached gases (except CO) and was injected into the column and electrophoresed (conditions: 20 kV, 0.01 M Na_2HPO_4, 0.006 M $Na_2B_4O_7$ pH 9.2). Heme-CO was also prepared according to the sample preparation procedures previously described and analyzed using CE. Two pieces of information were collected from injecting these samples individually: the raw solute retention times (T_r) and the corrected solute retention times (T_{corr}). The following equation corrects for changes in EOF from run to run:

$$T_{corr} = (T_r - T_o)/T_o$$

where T_o is the rate of EOF (the time at which the solvent in the sample plug reaches the detector) and T_r is the retention time of the solute. Inconsistencies in T_o may arise with an increased amount of solute injected into the column or from minor adsorption of solute onto the column wall over time. Therefore, values of T_{corr} are more useful than values of T_r when comparing several injections.

Reduced heme had an average retention time of 12.70 minutes. The reported average corrected retention time was 0.7072. The average retention time for heme-CO was 11.97 minutes. The average corrected retention time for heme-CO was 0.6150. The retention times consistently differed by

approximately 0.5 minutes, which is a considerable difference in a CE analysis. These results correspond to the theory of separation using CE. Although the molecular charges of reduced heme and heme-CO are similar in a basic medium, their molecular configurations are quite different. Reduced heme is more planar than heme-CO (12). Because it has a larger surface area, reduced heme is predicted to travel at a slower rate than heme-CO. The reported retention times and electropherograms reflected this prediction as heme-CO passed through the column more quickly.

The next goal in this experiment was to observe such a difference when a mixture of reduced heme and heme-CO was injected. When a 150 μL of heme-CO/reduced heme solution was electrophoresed under the same conditions as those used for the solutes run individually, two different signals were observed. When the heme-CO/reduced heme sample was spiked with 10 μL of reduced heme solution, an increase in the area of the signal corresponding to reduced heme was observed. An electropherogram representative of the results observed in many of these analyses is presented in Figure 3. When this spiked sample was further spiked with heme-CO, the reduced heme peak decreased in area relative to that of the heme-CO

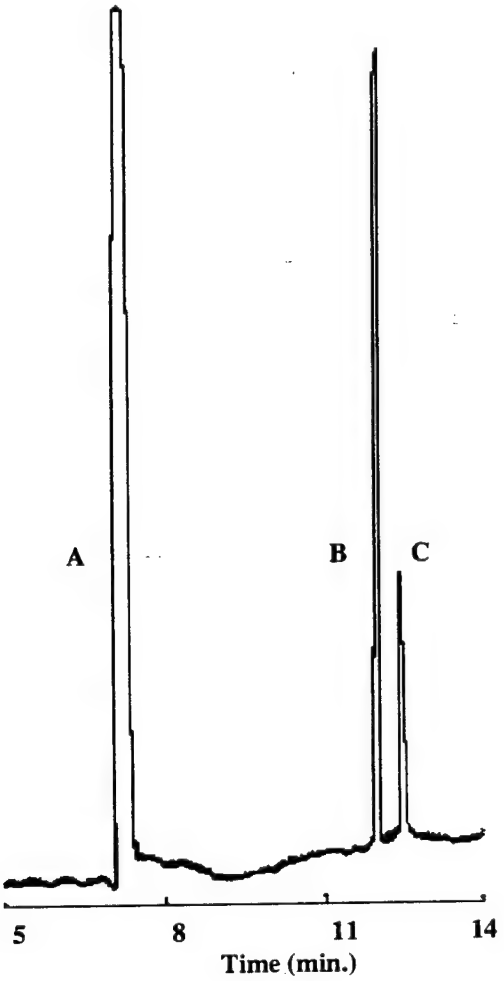

Figure 3: An electropherogram of a heme-CO sample spiked with reduced heme. Peak A represents acetone (T_0). Peak B represents heme-CO. Peak C represents reduced heme (50 cm effective, 50 µm i.d., 250 V/cm, 390 nm, 0.01 M Na_2HPO_4, 0.006 M $Na_2B_4O_7$, pH 9.2, 1.0 cm/min).

signal (due to sample dilution) and the heme-CO peak increased in area.

Blood Studies Using Capillary Electrophoresis

Prepared blood samples were injected into the column and electrophoresed (conditions: 20 kV, 0.01 M Na_2HPO_4,0.006 M $Na_2B_4O_7$ pH 9.2). Signals for reduced heme and heme-CO from these blood samples were observed. Two typical electropherograms representing results observed from samples that were analyzed multiple times over several days are presented in Figure 4. The average retention times of heme-CO and reduced heme from the two reported blood samples were 10.80 and 11.60 minutes, respectively. Many external factors, such as column age and slight changes in applied voltage, temperature, and pH, affected the retention times of runs on different days. Therefore, it was not surprising that the blood sample component retention times did not exactly reflect those reported for the standard Hb samples. However, a difference of 42-48 seconds in retention time is significant and was consistently observed.

The blood samples analyzed correspond to samples #1 and #2 from the **CO-oximetry/tonometry/GC Comparison Study** (see Table I). The results from the two CE analyses can be semi-

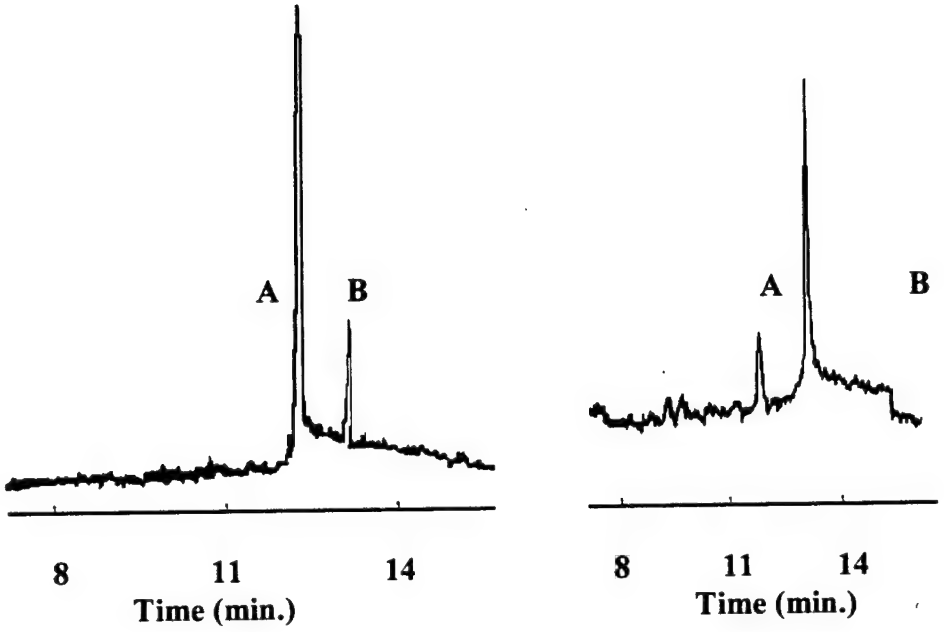

Figure 4: Electropherograms of blood samples #1 and #2. Peak A represents heme-CO. Peak B represents reduced heme (50 cm effective, 50 µm i.d., 250 V/cm, 390 nm, 0.01 M Na_2HPO_4, 0.006 M $Na_2B_4O_7$, pH 9.2, 1.0 cm/min).

quantitatively compared with those already reported from the CO-oximetry/tonometric/GC Comparison Study. From Table I, the % Hb-CO from the CO-oximeter analysis for blood samples #1 and #2 were 84.1 % and 0.100 %.

The %Hb-CO values from the tonometry/GC analysis for blood samples #1 and #2 were 80.24 % and 0.6800 %. The peak areas of the heme-CO from the CE analysis for blood samples #1 and #2 were 79.9 % and 7.9 %. The percent heme-CO for each sample was calculated by measuring the areas underneath the heme-CO and reduced heme peaks of an electropherogram. The percent heme-CO of this total area represents the % Hb-CO of the sample. It is encouraging that the results for blood sample #1 compare well with the current methods of CO analysis. However, the CE results for blood sample #2 do not compare well. The CE result of 7.9 % is a more realistic value of % Hb-CO than 0.1 % or 0.68 % based on the reported fact that the average person's blood naturally contains 3-10 % Hb-CO (14).

CONCLUSIONS

Significant progress has been made towards developing a method to analyze Hb-CO using CE. Sample preparation has been perfected. The CE conditions for this analysis have been determined using an in-house constructed CE instrument with a variable wavelength detector to analyze heme at its maximum wavelength of absorbance (390 nm). Standards that confirm peaks for reduced heme and heme-CO have been successfully tested. Qualitative and quantitative analyses of real blood samples have been successfully completed. The results from these analyses were compared with those from the existing methods of Hb-CO analysis. The results of this project indicate that CE might be used to determine more realistically lower concentrations of Hb-CO in blood.

The results of this project also present CE as a favorable candidate for CO analysis. CE partially solves the problems of sample requirements, analysis time, and reproducibility that exist with the current methods. In these CE analyses, 1 mL of blood was typically used in sample preparation and analysis. It is possible to use as little as 0.10 mL of sample for such a CE analysis. This amount is minute when compared to the several mL required for CO-

oximetry and tonometry/GC. Also, complete CE sample preparation and analysis time is, on average, 20 minutes as compared to the 45 minutes needed for the existing methods. Table I, which reports the difference in percent CO data between CO-oximetry and tonometry/GC, makes clear the reproducibility problems of the existing methods. CE was quite reproducible for the analyses of standard Hb samples. For example, the electropherograms that result from several injections of the same sample on the same day typically had retention times that differed only minimally (by ~6 seconds). Multiple analyses of a sample are best performed on the same day, as analyses after several days result in longer retention times (longer by up to 20 seconds). In analyzing blood samples, reproducibility was slightly diminished as a result of oxidation or degradation of the samples over time (the samples tested had been taken from victims several months prior to analysis). Ideally, one would analyze blood samples immediately after CO exposure is known to have occurred. Future studies to analyze more blood samples are necessary to optimize this method of analysis.

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